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**CORRECTED VERSIONS OF PAMPHLETS****VERSIONS CORRIGÉES DE BROCHURES**

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(Suite sur la page suivante)



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<p>(54) Title: <b>THERAPEUTIC COMPOSITIONS</b></p> <p>(57) Abstract</p> <p>Compositions comprising ketone bodies and/or their metabolic precursors are provided that are suitable for administration to humans and animals and which have the properties of, <i>inter alia</i>, (i) increasing cardiac efficiency, particularly efficiency in use of glucose, (ii) for providing energy source, particularly in diabetes and insulin resistant states and (iii) treating disorders caused by damage to brain cells, particularly by retarding or preventing brain damage in memory associated brain areas such as found in Alzheimer's and similar conditions. These compositions may be taken as nutritional aids, for example for athletes, or for the treatment of medical conditions, particularly those associated with poor cardiac efficiency, insulin resistance and neuronal damage. The invention further provides methods of treatment and novel esters and polymers for inclusion in the compositions of the invention.</p>		

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### THERAPEUTIC COMPOSITIONS.

The present invention relates to compositions suitable for administration to humans and animals which have the properties of, *inter alia*, (i) increasing cardiac efficiency, particularly efficiency in use of glucose, (ii) for providing energy source, particularly in  
5 diabetes and insulin resistant states and (iii) treating disorders caused by damage to brain cells, particularly by retarding or preventing brain damage in memory associated brain areas such as found in Alzheimer's and similar conditions. These compositions may be taken as nutritional aids, for example for athletes, or for the treatment of medical conditions, particularly those associated with poor cardiac efficiency, insulin resistance and  
10 memory loss. The invention further provides methods of treatment and novel esters and polymers for inclusion in the compositions of the invention.

Abnormal elevation of blood sugar occurs not only in insulin deficient and non insulin dependent diabetes but also in a variety of other diseases. The hyperglycaemia of diabetes results from an inability to metabolize and the over production of glucose. Both  
15 types of diabetes are treated with diet; Type I diabetes almost always requires additional insulin, whereas non-insulin dependent diabetes, such as senile onset diabetes, may be treated with diet and weight loss, although insulin is increasingly used to control hyperglycaemia.

Increased sympathetic stimulation or elevated glucagon levels, in addition to  
20 increasing glycogenolysis in liver, also stimulate free fatty acid release from adipocytes. After acute myocardial infarction or during heart failure, increased sympathetic nervous activity or administration of sympathomimetics accelerate glycogenolysis, decrease release of insulin from P cells of the pancreas and cause relative insulin resistance. While the importance of diet, or substrate availability, is taken as a given in the treatment of diabetes,  
25 the critical effects of substrate choice in insulin resistant states has not been widely appreciated or applied in clinical practice. Instead contemporary interest has focused upon the complex signalling cascade which follows the binding of insulin to its receptor. This increasingly complex cascade of messages involving protein tyrosine kinases and phosphatases, inositol and other phospholipids, while holding promise for the ultimate  
30 understanding of non-insulin dependent diabetes, has yet to provide significant new therapies for either diabetes or insulin resistance.

Leaving aside the longer term effects of insulin on growth, the acute metabolic effects of insulin have been thought to be accounted for by action at three major enzymatic steps in the conversion of glucose to CO<sub>2</sub>. Firstly insulin promotes the translocation of the glucose transporter, Glut4, from endoplasmic reticular to plasma membranes, thus  
5 increasing the transport of glucose from the extra to intracellular phase.(see refs. 1 and 2). Secondly, insulin increases the accumulation of glycogen. This has been attributed to dephosphorylation of glycogen synthase (3) by protein phosphatase 1. Thirdly, insulin stimulates the activity of mitochondrial pyruvate dehydrogenase multi-enzyme complex (4 and 5) through dephosphorylation by a Ca<sup>2+</sup> sensitive (6) intramitochondrial protein  
10 phosphosphatase.

An important, but poorly understood effect of insulin is its use in cardiac disease where in combination with glucose, potassium chloride and GIK, it improved electrocardiographic abnormalities accompanying myocardial infarction (7 and 8), and improved cardiac performance after post pump stunning (9). This treatment has been  
15 advocated recently for a number of other serious cardiac diseases (10 and 11). The beneficial effects of GIK infusion have been attributed to its ability to decrease free fatty acid release and improve membrane stability (12). However, other more recent work suggests more fundamental reasons. In heart cells that are anoxic, glucose is the only fuel capable of providing the ATP necessary to maintain viability (13).

Administration of glucose plus insulin would increase the availability of intracellular glucose providing a source of ATP production in the absence of O<sub>2</sub>. While this would explain certain beneficial effects, it would not account for the correction of EKG abnormalities nor the improved cardiac index in hearts treated with GIK because electrical activity and cardiac work requires actively respiring cardiac cells, not ones which are totally  
25 anoxic and therefore without electrical activity or the ability to perform mechanical work.

Understanding the enzymatic sites of insulin's action does not, by itself, define the effects of insulin deficiency upon the cellular metabolism or physiological function. How insulin acts at this larger level can best be understood by looking at the way nature deals with insulin deficiency. The natural compensation for decreased insulin during fasting is  
30 the accelerated hepatic conversion of the free fatty acids to the ketone bodies raising blood D-β-hydroxybutyrate and acetoacetate to about 6 mM. At these levels, ketones, rather than

glucose, become the substrate for most organs, including even the brain (14). Although mild ketosis is the normal response to decreased insulin, physicians fear ketone bodies because their massive overproduction can be life threatening in diabetic ketoacidosis.

The present inventor has previously compared the effect of physiological levels of ketone bodies to the metabolic and physiological effects of insulin, particularly comparing the insulin deficient working rat heart perfused with glucose alone, to hearts to which was added either 4 mM D- $\beta$ -hydroxybutyrate/1 mM acetoacetate, saturating doses of insulin or the combination and has shown how provision of simple substrates can mimic the effects of insulin in changing the concentrations of the intermediates of both glycolysis and the TCA cycle and thereby controlling the flux of glucose in this very specialised tissue. In addition he has determined that a primary but previously unrecognized effect of insulin or a ratio of ketones is to alter mitochondrial redox states in such a way so as to increase the  $\Delta G_{\text{ATPhydrolysis}}$  and with that, the gradients of inorganic ions between the various cellular phases and the physiological performance of heart.

The present application teaches that such ketone bodies can also provide a therapeutic approach to the treatment of insulin resistance where the normal insulin signalling pathway is disordered and in conditions where the efficiency of cardiac hydraulic work is decreased for metabolic reasons. The inventor has determined that use of ketone bodies has great advantage over use of insulin itself for reasons that will become evident from the description below, not least of these being the elimination of carbohydrate intake control otherwise necessary.

The present application further addresses the problem of neurodegenerative diseases, particularly disease where neurons are subject to neurotoxic effects of pathogenic agents such as protein plaques and further provides compositions for use in treating these and the aforesaid disorders.

Alzheimer's disease is a genetically heterogeneous group of progressively fatal neurological diseases characterized pathologically by accumulation of amyloid plaques in brain and clinically by impairment of recent memory leading to dementia and death. In addition to the cases of Alzheimer's disease linked to genetic causes, sporadic cases, without an apparent family history of the disease, also occur. For example pathological changes characteristic of Alzheimer's disease occur after head trauma (73) or after

inflammatory diseases stimulating production of the cytokine interleukin-1 (97).

The early symptom of the disease is loss of recent memory associated with impairment and death of cell in the hippocampus accounting for the early impairment of recent memory. Measurement of the hippocampal volumes using magnetic resonance  
5 imaging (MRI) shows that atrophy of hippocampus occurs prior to the clinical onset of memory loss and progresses with a loss of volume of about 8% per year during the 2 years over which symptoms first appeared (70).

The diagnosis of Alzheimer's disease is made clinically by this impairment in recent memory, associated with lesions in the hippocampal portion of the temporal lobe.  
10 Neuropathologically, the diagnosis depends upon the finding of neurofibrillary tangles within the cells, amyloid or senile plaques in the extracellular space and loss of neuronal number (61). The neurofibrillary tangles are comprised of paired hyperphosphorylated tau protein, whose usual function in the cell, when not phosphorylated, is to bind to and stabilize tubulin in its formation of microtubules within the cell. Hyperphosphorylation of  
15 tau is catalysed by glycogen synthase kinase 3 $\beta$ , among other kinases and dephosphorylated by protein phosphatase 2A-1, 2B or 1(108).

However, there is not necessarily a clear, bright line between the pathological brain changes and the memory deficits which occur prematurely in Alzheimer's disease and the pathological changes in brain anatomy and memory function which are found in the  
20 "normal" aging population. Rather the difference is a quantitative one dependent upon rate (94). Such changes in memory function in the normal aged are also accompanied by a decreased glucose tolerance signifying an inability to metabolize glucose. In such situations, treatments aimed at rectifying the pathophysiological processes of Alzheimer's disease, would be expected to be applicable to the correction of the metabolic effects  
25 associated with normal aging.

While Alzheimer's disease of the familial or the sporadic type is the major dementia found in the aging population, other types of dementia are also found. These include but are not limited to: the fronto-temporal degeneration associated with Pick's disease, vascular dementia, senile dementia of Lewy body type, dementia of Parkinsonism with frontal  
30 atrophy, progressive supranuclear palsy and corticobasal degeneration and Downs syndrome associated Alzheimers'. Plaque formation is also seen in the spongiform

encephalopathies such as CJD, scrapie and BSE. The present invention is directed to treatment of such neurodegenerative diseases, particularly those involving neurotoxic protein plaques, eg. amyloid plaques.

Many of these aforesaid apparently unrelated conditions have the  
5 hyperphosphorylated tau proteins found in Alzheimer's disease (69), opening up the possibility that the same kinase which phosphorylated tau would also phosphorylate the PDH complex producing a similar deficiency in mitochondrial energy production and acetyl choline synthesis found in Alzheimer's disease but involving other brain regions. The present inventor has determined that in this respect treatments applicable to  
10 Alzheimer's disease might be applied to these diseases as well. In addition, the inventor has determined that such treatment will also be applicable to peripheral neurological wasting diseases, such as myasthenia gravis and muscular dystrophy.

At present there is no effective treatment for Alzheimer's disease. Research efforts are focused on defining its genetic cause but to date there has been no succesful gene  
15 therapy. Genetic studies have linked Alzheimer's disease with Mongolism and in its early onset form to locus on chromosome 21 causing accumulation of amyloid precursor protein (APP)(73), a transmembrane glycoprotein existing in 8 isoforms. Numerous fragments of this protein are derived by proteolysis and the plaques characteristic of Alzheimer's disease have been shown to contain accumulation of the oligomer of  $\beta$  amyloid  
20 protein ( $A\beta_{1-42}$ ). An early onset autosomally dominant form of Alzheimer's disease has also been related to a presenilin 1 locus on chromosome 14.

A late onset form of Alzheimer's disease is associated with the type 4 allele of apolipoprotein E (69,98) on chromosome 19, although other workers suggest that this apparent correlation may be related instead of  $\alpha 1$  antichymotrypsin locus instead (100).  
25 All transgenic mice expressing increased amounts of amyloid precursor protein over 18 months of age showed hippocampal degeneration with many of the pathological characteristics of Alzheimer's disease (90).

The current status of knowledge on the defective genes and gene products in Alzheimer's disease has recently been summarized (Table 1 of ref. 96).

30

	Chromosome	Gene Defect	Age of Onset	A $\beta$ Phenotype
	21	$\beta$ APP mutations	50's	~ Production of total A $\beta$ peptides of A $\beta_{1-42}$
5	19	apoE4 polymorphism	60's or >	~ density of A $\beta$ plaques and vascular deposits
	14	Presenilin 1 mutations	40's & 50's	~ production of A $\beta_{1-42}$
	1	Presenilin 2 mutations	50's	~ production of A $\beta_{1-42}$

10 It is clear from the above table that the common phenotype associated with the genetic forms of Alzheimer's disease is the accumulation of the amyloid peptide A $\beta_{1-42}$  (96). It is this A $\beta_{1-42}$  which inactivates PDH thus impairing mitochondrial energy and citrate production in normally obligate glucose consuming tissue (95) and at the same time impairing synthesis of the critical neurotransmitter, acetyl choline (67,68). The application of A $\beta_{1-42}$  to neuronal cells is associated with the downregulation of the anti-apototic protein bcl-1 and increases levels of bax, a protein known to be associated with cell death (92). In addition to amyloid plaques comprised of A $\beta_{1-42}$ , neurofibrillary tangles comprised of hyperphosphorylated tau protein, and decreased brain acetyl choline levels, cell death is the fourth pathological characteristic of Alzheimer's disease. These pathological characteristics can be related, at least in part, to excess A $\beta_{1-42}$  and its inhibition of PDH.

20 Modest clinical improvement in symptoms can occur by treatment with acetyl choline esterase inhibitors (57), presumably by increasing cholinergic efferents originating in the septal nuclei and traversing Broca's diagonal band to hippocampus in the anterior portion of the limbic system of brain. However the progress in the molecular biology of Alzheimer's disease has caused the search for new therapies to concentrate upon four major areas (96): (i) protease inhibitors that partially decrease the activity of the enzymes ( $\beta$  and  $\gamma$  secretase) that cleave A $\beta$  ( $\beta$  amyloid fragments) from  $\beta$ APP ( $\beta$  amyloid precursor proteins); (ii) compounds that bind to extracellular A $\beta$  that prevent its cytotoxic effects; (iii) brain specific anti-inflammatory drugs that block the microglial (brain macrophages) activation, cytokine release, and acute phase response that occur in affected brain regions; and (iv) compounds such as antioxidants, neuronal calcium channel blocks, or antiapoptotic agents that interfere with the mechanisms of A $\beta$  triggered neurotoxicity.

The therapy which the present inventor now proposes differs from the four approaches listed above in that it bypasses the block in metabolic energy production

resulting from inhibition of PDH by  $\text{A}\beta_{1-42}$  by administering ketone bodies or their precursors. Neuronal cells are capable of metabolizing such compounds even in the presence of a deficiency of glucose, the normal energy substrate for brain (63). Because ketones can increase the  $\Delta G$  of ATP hydrolysis, the gradients of both intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$  will be increased, preventing cell death associated with increased intracellular  $\text{Ca}^{2+}$ . Furthermore, the increase in citrate generation by the Krebs cycle will provide, when translocated into cytoplasm, a source of cytoplasmic acetyl CoA required to remedy the deficiency of acetyl choline characteristic of Alzheimer's brains.

The elevation of blood ketones necessary to correct these metabolic defects can be accomplished by parenteral, enteral means or dietary means and does not require the administration of potentially toxic pharmacological agents.

There has been long experience with ketogenic diets in children treated for epilepsy. Such diets are however unsuitable for use in adults due to adverse effects on the circulatory system. The present inventions application of ketone bodies should provide all the therapeutic effects of such diet, which is not itself found to be toxic in children, with none of the side effects that render it unused adults. Furthermore, the inventor has determined that with the correction of the aforesaid metabolic defects, cytokine responses and the increase in apoptotic peptides in degenerating cells will decrease due to the increase in neuronal cell energy status and the increased trophic stimulation resulting from increased acetyl choline synthesis.

Since the priority date of this application, EP 0780123 A1 has been published which relates to use of acetoacetate,  $\beta$ -hydroxybutyrate, monhydric, dihydric or trihydric alcohol esters of these or oligomers of  $\beta$ -hydroxybutyrate for suppressing cerebral edema, protecting cerebral function, rectifying cerebral energy metabolism and reducing the extent of cerebral infarction. It should be noted however, that it has been known since 1979 that sodium hydroxybutyrate increases cerebral circulation and regional vasomotor reflexes by up to 40% (Biull.Eksp.Biol.Med Vol 88 11, pp555-557). The treatment that the present inventor now provides goes beyond such effects on circulation as it provides treatment for cells that are unable to function due to neurodegeneration, eg caused by neurotoxic agents such as peptides and proteins, and genetic abnormality. The treatment involves action of ketone bodies on the cells themselves and not the flow of blood to them.

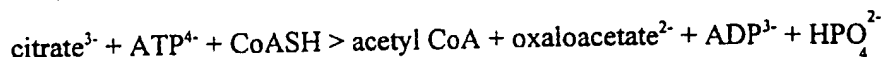
In reducing this invention to practice the inventor has further determined that ketone bodies, provided by direct administration or by administration of their metabolic precursors in amounts sufficient to raise total blood ketone body concentration to elevated levels result in more than simple maintenance of cell viability but actually improve cell function and growth beyond that of normal, ie. control levels in a manner unrelated to blood flow or nutrition. In this respect the invention further provides use of ketone bodies as nerve stimulant factors, ie. nerve growth factors and factors capable of stimulating enhanced neuronal function, such as increase of metabolic rate and increase of extent of functional features such as axons and dendrites. This aspect of the present invention offers a mechanism for improvement of neuronal function as well as mere retardation of degradation.

The recent work of Hoshi and collaborators (77, 78) strongly suggests that a part of the amyloid protein whose accumulation is the hallmark of Alzheimer's disease,  $A\beta_{1-42}$ , acts as a mitochondrial histidine protein kinase which phosphorylates and inactivates the pyruvate dehydrogenase multienzyme complex. The PDH complex is a mitochondrial enzyme responsible for the generation of acetyl CoA and NADH from the pyruvate produced by glycolysis within the cytoplasm. The mitochondrial acetyl CoA formed condenses with oxaloacetate to start the Krebs TCA cycle completely combusting pyruvate to  $CO_2$  while providing the mitochondria with the reducing power which becomes the substrate for the electron transport system through which the energy required for mitochondrial ATP synthesis is generated. PDH thus stands at the crossroads of the two major energy producing pathways of the cell, glycolysis and the Krebs cycle, and clearly serves a critical function in living cells.

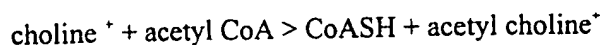
There are two major consequences of the inhibition of PDH. Firstly, in neuronal tissues, which under normal metabolic conditions are totally dependent upon glucose for energy production, inhibition of PDH results in a lowered efficiency of energy production, a lowered energy of hydrolysis of ATP, a decrease in both acetyl CoA and the metabolites of the first 1/3 of the TCA cycle and a deficiency of mitochondrial NADH (95). A decrease in the energy of ATP hydrolysis leads to increased intracellular  $Na^+$  and  $Ca^{2+}$ , loss of cellular  $K^+$  and ultimately cell death (86). Hippocampal cells, critical for the fixation of recent memories, are particularly sensitive to a number of forms of injury, and the death of

these cells is the hallmark both clinically and pathologically of Alzheimer's disease.

A second major consequence of PDH inhibition is a deficiency of mitochondrial citrate (95). Citrate, or one of its metabolites, is exported to the cytoplasm from mitochondria where it is converted to cytosolic acetyl CoA by ATP citrate lyase  
 5 (EC 4.1.3.8) in the reaction:



The acetyl CoA then combines with choline through the action of choline acetyl transferase (EC 2.3.1.6) to form acetyl choline in the reaction:



10 Neuronal culture of septal cells exposed to 1  $\mu\text{M}$   $\text{A}\beta_{1-42}$  for 24 hours showed a decrease in acetyl choline production of over five fold (78) with no decrease in the activity of choline acetyl transferase. The inferred cause of this decreased production was a deficiency of acetyl CoA due to inhibition of the PDH complex caused by activation of the *TPK1/GSK-3\beta* protein kinase and subsequent phosphorylation of PDH (77).

15 As explained above isolated working hearts perfused with 10 mM glucose alone without insulin are inefficient and have impaired mitochondrial energy production. This defect in cellular energy production can be completely reversed by the provision of a physiological ratio of ketone bodies consisting of 4 mM D- $\beta$  hydroxybutyrate and 1 mM acetoacetate (95). Brain was thought to be capable of using only glucose as its metabolic  
 20 energy source and to be insensitive to the actions of insulin. However, in a remarkable clinical study performed in 1967, George Cahill and his collaborators (47) showed that up to 60% of the brain's need for metabolic energy could be met by ketone bodies in obese patients undergoing prolonged fasting. Even more remarkably, Cahill showed that administration of insulin to these patients in doses sufficient to drop their blood sugar from  
 25 4 to under 2 mM was associated with no impairment of mental functions in these patients whose blood D- $\beta$  hydroxybutyrate was 5.5 mM and acetoacetate 2 mM (see Figure 3 from ref 63). Clearly, when ketone bodies are present in the blood at levels above 5 mM, they are able to substitute for the brain's usual need for glucose and abolish the hypoglycemic symptoms expected at blood glucose levels of 1.5 mM

30 Ketone body utilization in brain is limited by the transport, with lesser utilization occurring in the basal ganglion at blood levels below 1 mM (76). However, at levels of

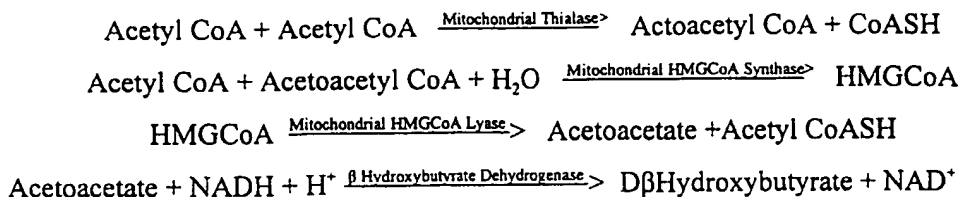
7.5 mM achieved in normal man by prolonged fasting, the rate of ketone body entry into brain is sufficient to take over the majority of cerebral energy needs and to prevent hypoglycemic symptoms, even in the face of blood sugar levels which would normally cause convulsions or coma (63) .

5 It is the inventors hypothesis that in Alzheimer's disease, where there is a block at PDH which prevents the normal energy production from glucose, if one can provide elevated, eg. normal fasting levels of ketones, one can bypass the PDH blockade present in these patients thereby preventing cell death due to energy depletion or lack of cholinergic stimulation and thus slow the progression of the memory loss and dementia.

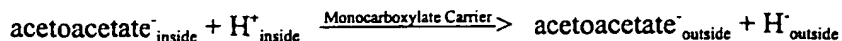
10 Furthermore, utilising the nerve growth/stimulatory effects of the ketone bodies, particularly D-β-hydroxybutyrate or a physiological ratio of this with acetoacetate, cells that are still viable can be caused to improve beyond the state to which they have degenerated and accordingly some improvement of function will be seen in patients.

In fed animals and in man the liver content, which is essentially that of blood, of  
15 acetoacetate is very low at 0.09 mM and D-β hydroxybutyrate is 0.123mM but rises after a 48 hour fast to 0.65 mM acetoacetate and 1.8 mM D-β hydroxybutyrate (84). The ketone bodies rise in starvation because the fall in insulin decreases the re-esterification of fatty acids to triglyceride in adipose tissue causing the release of free fatty acids into the blood stream. The released free fatty acids can then be taken up and used as a source of energy  
20 by muscle, heart, kidney and liver in the process of β oxidation. Liver, however, has the capacity to convert the free fatty acids to a metabolic fuel, ketones, for use by extrahepatic organs, including the brain, as an alternative to glucose during periods of fasting. The hepatic synthesis of ketone bodies occurs from mitochondrial acetyl CoA generated during the β-oxidation of fatty acids by liver in the following set of reactions:

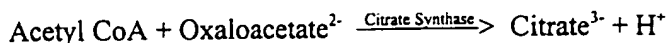
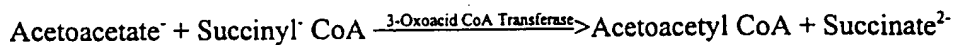
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30 Once made in the liver, ketone bodies are transported out of the liver into the blood stream by the monocarboxylate -H' co-transporter (20) by the following reaction:



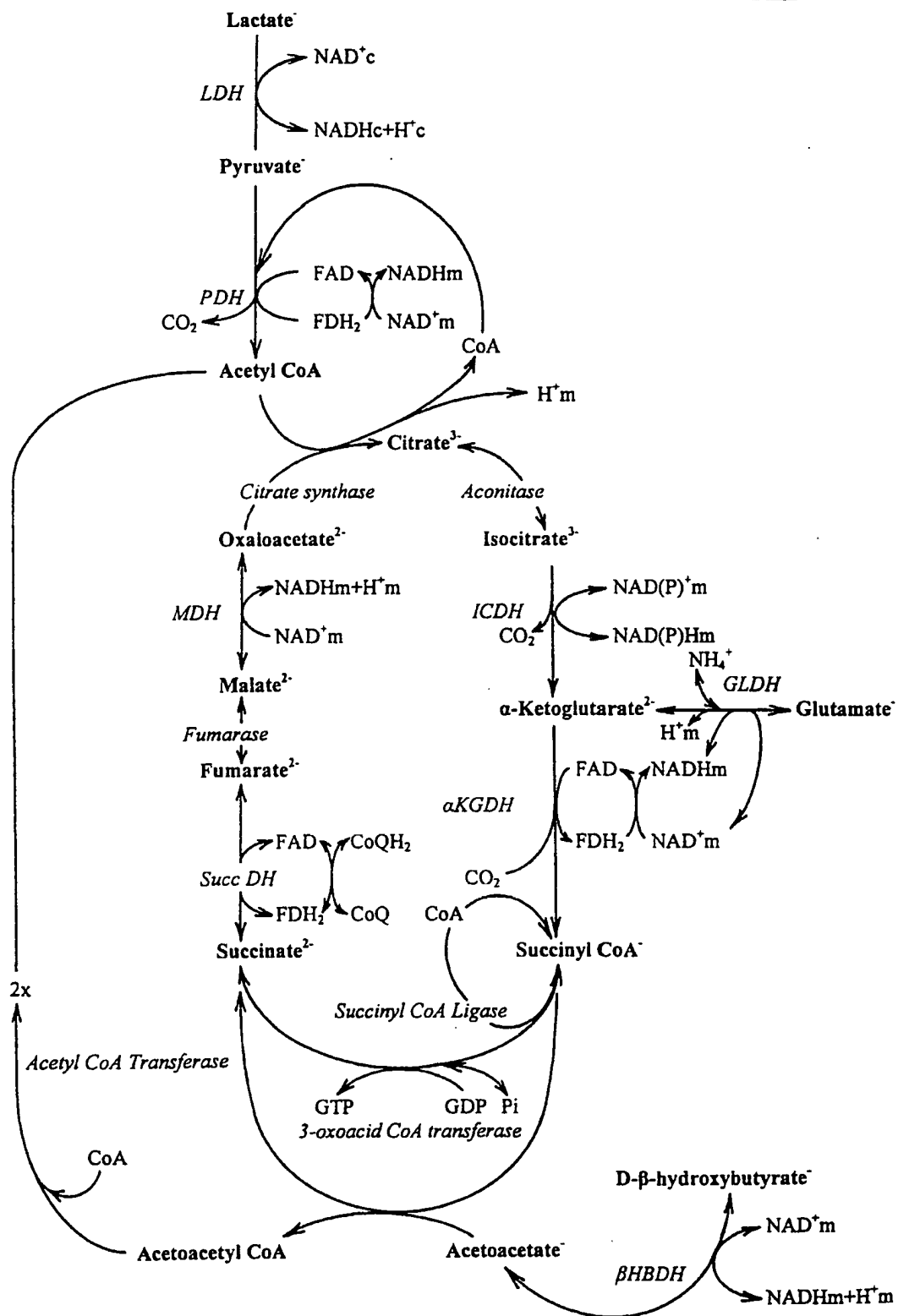
The ketone bodies enter extra-hepatic tissues on the same carrier, where other monocarboxylates can act as competitive inhibitors. Unphysiological isomers such as D-lactate or L-β-hydroxybutyrate can also act as competitive inhibitors to ketone body transport. Since ketone body transport across the blood brain barrier is the limiting factor to ketone body utilization in brain (76) every effort should be made to keep the blood concentration of these unphysiological enantiomers at low levels during ketogenic therapy. When blood ketone body concentrations are elevated to levels found in starvation, heart, muscle, kidney and brain utilize ketone bodies as the preferred energy substrate:



The present inventor has thus determined that the mitochondrial acetyl CoA from ketone bodies can thus replace the acetyl CoA deficiency which occurs during inhibition of PDH multienzyme complex in tissues dependent upon the metabolism of glucose for their supply of metabolic energy. The mitochondrial citrate supplied can also be transported to cytoplasm by the tri or dicarboxylic acid transporter where it can be converted to cytoplasmic acetyl CoA required for the synthesis of acetyl choline. The reactions of the Krebs cycle are shown in Scheme 1 to help illustrate these concepts further.

The liver cannot utilize ketone bodies because it lacks the 3 Oxoacid CoA transferase necessary for the formation of acetoacetyl CoA. Ketone bodies, in contrast to free fatty acids, cannot produce acetyl CoA in liver. Since acetyl CoA is the essential precursor of fatty acid synthesis through malonyl CoA and cholesterol synthesis through cytosolic HMG CoA, ketone bodies cannot result in either increased fatty acid or cholesterol synthesis in liver, which usually accounts for over half of the bodies synthesis of these two potentially pathogenic materials. Liver is sensitive to the ratio of acetoacetate /D-β-hydroxybutyrate presented to it and will alter its mitochondrial free [NAD<sup>+</sup>]/[NADH], because of the near equilibrium established by β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (55)

**SCHEME 1**



The easiest way to increase blood ketones is starvation. On prolonged fasting blood ketones reach levels of 7.5 mM (62, 63). However, this option is not available on a long term basis, since death routinely occurs after a 60 day fast.

The ketogenic diet, comprised mainly of lipid, has been used since 1921 for the  
5 treatment of epilepsy in children, particularly myoclonic and akinetic seizures (109) and has proven effective in cases refractory to usual pharmacological means (71). Either oral or parenteral administration of free fatty acids or triglycerides can increase blood ketones, provided carbohydrate and insulin are low to prevent re-esterification in adipose tissue. Rats fed diets comprised of 70% corn oil, 20% casein hydrolysate, 5% cellulose,  
10 5% McCollums salt mixture, develop blood ketones of about 2 mM. Substitution of lard for corn oil raises blood ketones to almost 5 mM (Veech, unpublished).

An example of a traditional 1500/day calorie ketogenic diet recommended by the Marriott Corp. Health Care Services, Pediatric Diet Manual, Revised August 1987 as suitable for a 4-6 year old epileptic child contained from 3:1 to 4:1 g of fat for each g of  
15 combined carbohydrate and protein. At each of 3 meals the patient must eat 48 to 50 g fat, only 6 g protein and 10 to 6.5 g carbohydrate. In practice this means that at each meal the child must eat 32 g of margarine per day (about ¼ stick) and drink 92 g of heavy cream (about 100 ml), comprised mainly as medium chain length triglycerides.

An example of a diet achieving a 3:1 ratio of fat to combined carbohydrate and  
20 protein is given in Table 1 below.

Table 1. Sample 1500 calorie diet to achieve 3:1 lipid to carbohydrate + protein diet

	Amount (g)	Fat (g)	Protein (g)	CHO (g)
Breakfast				
Egg	32	4	4	
apple juice	70			7
margarine	11	10		
heavy cream	92	34	2	3
Total Breakfast		48	6	10
Lunch				
lean beef	12	1.75	3.5	
cooked carrots	45		0.6	3
canned pears	40			4
margarine	14	12.5		
heavy cream	92	34	2	3
Total Lunch		48.25	6.1	10
Supper				
Frankfurter	22.5	6	3	
Cooked broccoli	50		1	2
Watermelon	75			5
Margarine	8	7.5		
Heavy cream	92	34	2	3
Total Supper		47.5	6	10
Daily Total		143.75	18.1	30

In general the levels of ketone bodies achieved on such diets are about 2 mM  
 5 D- $\beta$  hydroxybutyrate and 1 mM acetoacetate while the levels of free fatty acids about 1

mM. Other variations of composition have been tried including medium chain length triglycerides. In general compliance with such restricted diets has been poor because of their unpalatability (56). High lipid, low carbohydrate diets also have been tried as therapeutic agents in cancer patients to reduce glucose availability to tumors (88) as weight  
5 reducing diets in patients with and without diabetes (74, 112) to improve exercise tolerance (83).

The limitation of diets which rely upon lipid to raise blood ketones to neurologically effective levels are many. Firstly, levels of ketone bodies on lipid based diets tend to be below 3 mM, significantly lower than the level of 7.5 mM achieved in  
10 normal obese humans during prolonged fasting. Secondly, unauthorized ingestion of carbohydrate increases insulin secretion and causes a rapid decrease in the hepatic conversion of free fatty acids to ketones with a consequent drop in blood ketones and the diversion of lipid to esterified to triglycerides by adipose tissue. Many anecdotal reports relate the resumption of seizures in children who "broke their diet with birthday cake".  
15 Thirdly the unpalatability and the necessity to avoid carbohydrate to sustain high ketone body levels makes such high lipid diets difficult to use in adults in an out patient setting, particularly in societies where traditionally high intake of refined sugars, bread, pasta, rice and potatoes occurs. In practice, the traditional high ketone diet cannot be enforced in patients, other than children beyond the age where all food is prepared at home under strict  
20 supervision. Fourthly, ingestion of such large amounts of lipid in the adult population would lead to significant hypertriglyceridemia with its pathological sequelae of increased vascular disease and sporadic hepatic and pancreatic disease, and therefore could not be prescribed on medical grounds. Ingestion of high lipid, low carbohydrate diets were popular in the 1970s for weight reduction in the face of high caloric intake, provided that  
25 carbohydrate intake was low. However, because of the increased awareness of the relationship of elevated blood lipids to atherosclerosis the popularity of this diet dropped abruptly.

Supplementing a liquid diet with 47% of its caloric content with either glucose or racemic 1,3 butandiol caused the blood ketone concentration to rise about 10 fold  
30 to 0.98 mM D- $\beta$  hydroxybutyrate and 0.33 mM acetoacetate (107). These values are slightly less than obtained normally in a 48 hour fast and far below the levels of 7.5 mM

obtained in fasting man. Racemic 1,3 butandiol is converted by liver to acetoacetate and both the unnatural L- $\beta$  and the natural D- $\beta$  hydroxybutyrate (respectively (S) 3-hydroxybutanoate and (R) 3-hydroxybutanoate). Although racemic 1,3 butandiol has been extensively studied as a cheap caloric source in animal food and has even been used  
5 experimentally in human diets (81, 101) the production of the unnatural L-isomer is likely in the long run to produce significant toxicity as has been shown for the human use of the unnatural D-lactate (64). One disadvantage of administering the unnatural L isomer is that it competes for transport with the natural D- $\beta$  hydroxybutyrate. Thus provision of the (R) 1,3 butandiol as a precursor of ketone bodies is one possibility that avoids unnecessary  
10 administration or production of the unnatural isomer.

The mono and diester of racemic 1,3 butandiol have been suggested as a source of calories and tested in pigs (67). Oral administration of a bolus of a diet containing 30% of calories as the esters produced brief peaks blood ketones to 5 mM. However, the use of racemic 1,3 butandiol with its production of the abnormal (S) 3-hydroxybutanoate is not  
15 to be recommended for the reasons stated above.

While use of racemic 1,3 butandiol in such formulations is not recommended, the esters of (R) 1,3 butandiol can be used, either alone or as the acetoacetate ester. (R) 1,3 butandiol may easily be synthesized by reduction of the monomeric D- $\beta$  hydroxybutyrate, with for example  $\text{LiAlH}_4$ . (R) 1,3 butandiol is subject to being  
20 oxidized in the liver to form D- $\beta$  hydroxybutyrate without marked distortion of the hepatic redox state. Studies in rats have shown that feeding racemic 1,3 butandiol caused liver cytosolic  $[\text{NAD}^+]/[\text{NADH}]$  to decrease from 1500 to about 1000 (87). By comparison, administration of ethanol reduces hepatic  $[\text{NAD}^+]/[\text{NADH}]$  to around 200 (106).

Acetoacetate, when freshly prepared, can be used in infusion solutions where it can  
25 be given in physiologically normal ratios to optimum effect (95). Because of manufacturing requirements which currently require long shelf life and heat sterilized fluids, acetoacetate has frequently been given in the form of an ester. This has been done to increase its shelf life and increase its stability to heat during sterilization. In the blood stream, esterase activity has been estimated to be about 0.1 mmol/min/ml and in liver  
30 about 15 mmol/min/g (68). In addition to esters combining 1,3 butandiol and acetoacetate there has also been extensive study of glycerol esters of acetoacetate in parenteral (59) and

enteral nutrition (82). Such preparations were reported to decrease gut atrophy, due to the high uptake of acetoacetate by gut cells and to be useful in treatment of burns (85).

However, neither 1,3 butandiol, which forms acetoacetate, nor glycerol, which is a precursor of glucose, is part of the normal redox couple, D- $\beta$  hydroxybutyrate/acetoacetate.

5 For the present invention, under optimum conditions, a physiological ratio of ketones should be given. If it is not, in the whole animal, the liver will adjust the ratio of ketones in accordance with its own mitochondrial free  $[NAD^+]/[NADH]$ . If an abnormal ratio of ketones is given pathological consequences are a distinct possibility. In the working heart, perfusion with acetoacetate as sole substrate, rapidly induces heart failure (99) in contrast  
10 to rat hearts perfused with a mixture of glucose, acetoacetate and D- $\beta$  hydroxybutyrate, where cardiac efficiency was increased by a physiological ratio of ketone bodies (95).

The best exogenous source of ketone bodies, which do not require ingestion of large amounts of lipid nor the use of material which produce the physiologically incompatible isomers L- $\beta$ -hydroxybutyrate would be ketone bodies themselves. However the present  
15 invention also provides alternatives for administration in therapy.

A first alternative are polyesters of D- $\beta$ -hydroxybutyrate. Natural polyesters of D- $\beta$ -hydroxybutyrate are sold as articles of commerce at polymers of 530,000 MW from *Alcaligenes eutrophus* (Sigma Chemical Co. St. Louis) or as 250,000 MW polymers for sugar beets (Fluka, Switzerland). The bacteria produce the polymer as a source of stored  
20 nutrient. The fermentation of these polymers by bacteria was developed in the 1970s by ICI in the UK and Solvay et Cie in Belgium, as a potentially biodegradable plastic for tampon covers and other uses. The system responsible for the synthesis of the poly D- $\beta$ -hydroxybutyrate has now been cloned and variations in the composition of the polymer produced, based on the substrates given to the bacteria demonstrated. However, these  
25 polymers failed to be able to compete with petroleum based plastics. Nevertheless the genes responsible for the synthesis of polyalkanoates has been cloned and expressed in a number of micro-organisms (93, 102, 113) allowing for production of this material in a variety of organisms under extremely variable conditions.

Poly D- $\beta$ -hydroxybutyrate comes in a number of forms from different biological  
30 sources as an insoluble white powder with little taste and no odour and is suitable for incorporation into compositions for oral or other means of administration. Esterases

capable of breaking the ester bonds of this material are ubiquitous in plasma and most cells. These polymer are also easily split by alkaline hydrolysis in vitro to make a series of polymers culminating in the production of the monomer of MW 104, which is transported from gut to portal vein by the normal monocarboxylate transporter. Alternatively acid  
5 hydrolysis may be carried out using the published method referred to in the Fluka promotional material.

Preferred forms of D- $\beta$ -hydroxybutyrate polymer are oligomers of that ketone body designed to be readily digestible and/or metabolised by humans or animals. These preferably are of 2 to 100 repeats long, typically 2 to 20 and most conveniently from 3 to  
10 10 repeats long. It will be realised that mixtures of such oligomers may be employed with advantage that a range of uptake characteristics might be obtained.

Once the monomer is in the blood stream, and since liver is incapable of metabolizing ketone bodies but can only alter the ratio of D- $\beta$ -hydroxybutyrate/acetoacetate, the ketone bodies are transported to extrahepatic tissues  
15 where they can be utilized. The blood levels of ketones achieved are not be subject to variation caused by noncompliant ingestion of carbohydrate, as is the case with the present ketogenic diet. Rather, they would simply be an additive to the normal diet, given in sufficient amounts to produce a sustained blood level, typically of between .0.3 to 20mM, more preferably 2 to 7.5 mM, over a 24 hour period, depending upon the condition being  
20 treated. In the case of resistant childhood epilepsy, blood levels of 2 mM are currently thought to be sufficient. In the case of Alzheimer's disease, attempts could be made to keep levels at 7.5 mM achieved in the fasting man studies, in an effort to provide alternative energy and acetyl CoA supplies to brain tissue in Alzheimer's patients where PDH capacity is impaired because of excess amounts of A $\beta_{1-42}$  amyloid peptide (77, 78).

25 The determination by the inventor that D- $\beta$ -hydroxybutyrate and its mixtures with acetoactetate act as a nerve stimulant, eg. nerve growth stimulant and/or stimulant of axon and dendritic growth, opens up the option of raising ketone body levels to lesser degrees than required nutritionally in order to treat neurodegeneration.

Compositions of the invention are preferably sterile and pyrogen free, particularly  
30 endotoxin free. Secondly, they are preferably formulated in such a way that they can be palatable when given as an additive to a normal diet to improve compliance of the patients

in taking the supplements. The oligomers and polymers are generally taste and smell free. Formulations of D- $\beta$ -hydroxybutyrate and its mixtures with acetoacetate may be coated with masking agents or may be targeted at the intestine by enterically coating them or otherwise encapsulating them as is well understood in the pharmaceuticals art.

5        Since ketone bodies contain about 6 calories/g, there is preferably a compensatory decrease in the amounts of the other nutrients taken to avoid obesity.

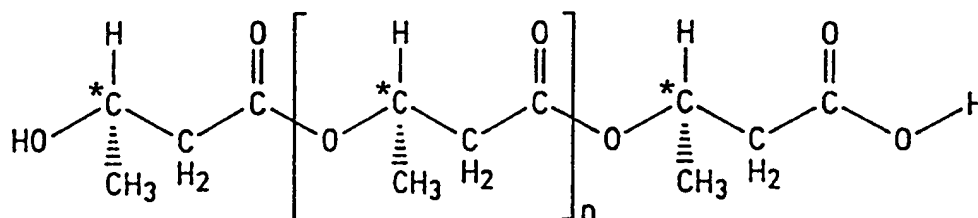
Particular advantages of using the ketone bodies or precursors such as poly or oligo-D- $\beta$ -hydroxybutyrate or acetoacetate esters are:

- 1) they can be eaten with a normal dietary load of carbohydrate without  
10        impairing its effects,
- 2) they will not raise blood VLDL, as with current cream and margarine containing diets, thus eliminating the risk of accelerated vascular disease, fatty liver and pancreatitis,
- 3) they will have a wider range of use in a greater variety of patients,  
15        including: type II diabetes to prevent hypoglycemic seizures and coma, in Alzheimer's disease and other neurodegenerative states to prevent death of nerve cells eg. hippocampal cells, and in refractory epilepsy due to either decreases in cerebral glucose transporters, defects in glycolysis, or so called Leigh's syndromes with congenital defects in PDH.

20        The second group of particular alternatives are acetoacetate esters of D- $\beta$ -hydroxybutyrate. Esters which provide a physiological ratio of acetoacetate to D- $\beta$ -hydroxybutyrate are preferred eg. from 1:1 to 1:20, more preferably from 1:1 to 1:10. The tetramer of D- $\beta$ -hydroxybutyrate with a terminal acetoacetate residue is particularly preferred. Such materials have the added virtue of having a physiological ratio of D- $\beta$ -  
25        hydroxybutyrate/acetoacetate moieties, thus removing the burden on liver of having to adjust the redox state of the administered nutrient without inducing abnormal reduction of hepatic  $[NAD^+]/[NADH]$  as occurs with excessive alcohol consumption. The polymeric esters, depending upon their length, have decreasing water solubility, but are heat stable. Such polymers can for example be used in oral and parenteral use in emulsions, whereas  
30        acetoacetate, in the unesterified state, is less preferred as it is subject to spontaneous decarboxylation to acetone with a half time at room temperature of about 30 days.

Examples of poly D-β-hydroxybutyrate or terminally oxidized poly D-β-hydroxybutyrate esters useable as ketone body precursors are given below.

Poly (R) 3-Hydroxybutyric acid

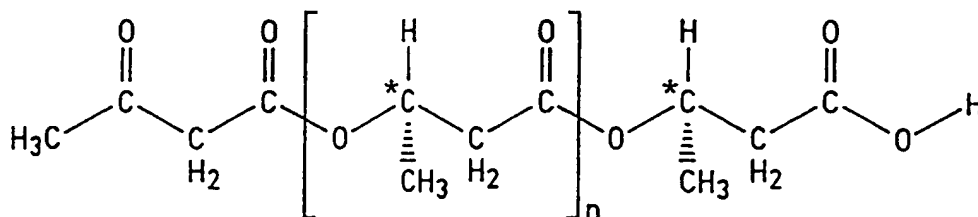


Terminal  
(R) 3-Hydroxy-  
butyric acid residue

(R) 3-Hydroxy-  
butyryl  
residue(s)

Terminal  
(R) 3-Hydroxy-  
butyric acid residue

Oxidized poly (R) 3-Hydroxybutyric acid



Acetoacetate  
residue

(R) 3-Hydroxy-  
butyryl  
residue(s)

Terminal  
(R) 3-Hydroxy-  
butyric acid residue

20

In each case n is selected such that the polymer or oligomer is readily metabolised on administration to a human or animal body to provide elevated ketone body levels in blood. Preferred values of n are integers of 0 to 1,000, more preferably 0 to 200, still more preferably 1 to 50 most preferably 1 to 20 particularly conveniently being from 3 to 5.

A number of variations of this material, including the polyester D-β-hydroxybutyrate itself can also be tried for suitable manufacturing characteristics. The material is a tasteless white powder. After partial alkaline hydrolysis, a mixture of varying

chain length polymers would be provided, which would tend to smooth gut absorption and maintain high sustained levels of ketone over a 24 hour period.

Treatment may comprise provision of a significant portion of the caloric intake of patients with the D- $\beta$ -hydroxybutyrate polyester formulated to give retarded release, so as  
5 to maintain blood ketones in the elevated range, eg. 0.5 to 20 mM, preferably 2-7.5 mM, range over a 24 hour period. Release of the ketone bodies into the blood may be restricted by application of a variety of techniques such as microencapsulation, adsorption and the like which is currently practised in the oral administration of a number of pharmaceutical agents. Enetrically coated forms targeting delivery post stomach may be particularly used  
10 where the material does not require hydrolysis in acid environment. Where some such hydrolysis is desired uncoated forms may be used. Some forms may include enzymes capable of cleaving the esters to release the ketone bodies such as those referred to in Doi. Microbial Polyesters.

Intravenous infusion of sodium salts of D- $\beta$ -hydroxybutyrate has been performed  
15 on normal human subjects and patients for a number of conditions, eg. those undergoing treatment for severe sepsis in an intensive care unit. It was found to be non-toxic and capable of decreasing glucose free fatty acids and glycerol concentration, but ineffective in decreasing leucine oxidation.

The monomer of D- $\beta$ -hydroxybutyrate is a white, odourless crystal with a slightly  
20 tart or acid taste which is less in intensity in comparison to vinegar or lemon juice. It can be formulated into most foodstuffs, eg. drinks, puddings, mashed vegetables or inert fillers. The acid forms of D- $\beta$ -hydroxybutyrate are suitable for use orally as they have a pKa of 4.4. This is less acid than citric acid with pKa1 of 3.1 and pKa2 of 4.8 and slightly more acidic than acetic acid with a pKa of 4.7.

25 Preferably, only the natural D- or (R) isomer is used in this formulation. Since in practice it is not possible to achieve absolute isomeric purity, the article of commerce currently sold by Sigma, St. Louis MO or Fluka, Ronkonkoma, NY is the most suitable for this purpose. The optical rotation of the commercially available D- $\beta$ -hydroxybutyric acid is  $-25^{\circ} \pm 1$  at the wavelength of Na and its melting point  $43-46^{\circ}\text{C}$ . The optical rotation of  
30 the Na salt of D- $\beta$ -hydroxybutyrate is  $-14.5^{\circ}$  and its melting point  $149-153^{\circ}\text{C}$ . Both can be assayed by standard enzymatic analysis using D- $\beta$ -hydroxybutyrate dehydrogenase (EC

1.1.1.30)(5). Acetoacetate can be determined using the same enzyme (56). The unphysiological (S) isomer is not measurable with enzymatic analysis but can be measured using GC mass spec (13).

For a 1500 calorie diet, the human adult patient could consume 198 g of ketones per day. For a 2000 calorie diet of the same proportions, one could consume 264 g of ketones per day. On the ketogenic lipid diet blood ketones are elevated to about 2 mM. On the ketone diet, ketone levels should be higher because ketones have been substituted at the caloric equivalent of fat, that is 1.5 g of ketone/1 g of fat. Accordingly, blood ketones should be approximately 3 mM, but still below the level achieved in fasting man of 7.5 mM.

The advantage of using ketone bodies themselves are several. Firstly, provision of ketone bodies themselves does not require the limitation of carbohydrate, thus increasing the palatability of the dietary formulations, particularly in cultures where high carbohydrate diets are common. Secondly, ketone bodies can be metabolized by muscle, heart and brain tissue, but not liver. Hence the fatty liver, which may be an untoward side effect of the ketogenic diet, is avoided. Thirdly, the ability to include carbohydrate in the dietary formulations increases the chance of compliance and opens up practical therapeutic approaches to type II diabetics where insulin is high, making the known ketogenic diet unworkable.

The present inventor has determined that, while any elevation of ketone bodies may be desirable, a preferred amount of ketone bodies to be administered will be sufficient to elevate blood levels to the 0.5 to 20mM level, preferably to the 2mM to 7.5mM level and above, particularly when attempting to arrest the death of brain cells in diseases such as Alzheimer's. While dead cells cannot be restored, arrest of further deterioration and at least some restoration of function is to be anticipated.

Thus in a first aspect of the present invention there is provided the use acetoacetate, D- $\beta$ -hydroxybutyrate or a metabolic precursor of either in the manufacture of a medicament or nutritional aid (i) for increasing cardiac efficiency, particularly efficiency in use of glucose (ii) for providing energy source, particularly in treating diabetes and insulin resistant states or by increasing the response of a body to insulin (iii) for reversing, retarding or preventing nerve cell damage or death related disorders, particularly

neurodegenerative disorders such as memory associated disorders such as Alzheimer's, seizure and related states such as encephalopathies such as CJD and BSE.

The term metabolic precursor thereof particularly relates to compounds that comprise 1,3-butanediol, acetoacetyl or D- $\beta$ -hydroxybutyrate moieties such as acetoacetyl-  
5 1,3-butanediol, acetoacetyl- D- $\beta$ -hydroxybutyrate, and acetoacetyl glycerol. Esters of any such compounds with monohydric, dihydric or trihydric alcohols is also envisaged.

This aspect includes such use as a neuronal stimulant eg capable of stimulating axonal and/or dendritic growth in nerve cells, eg. in Hippocampus or Substantia nigra particularly in diseases where neurogeneration has serious clinical consequences.

10 In diabetic patients this use of these compounds allows maintenance of low blood sugar levels without fear of hypoglycemic complications. In normal non-diabetic subjects the fasting blood sugar is 80 to 90 mg % (4.4-5mM) rising to 130mg % (7.2mM) after a meal. In diabetics 'tight control' of diabetes has long been recommended as a method for retardation of vascular complications but, in practice, physicians have found it difficult to  
15 keep blood sugars tightly controlled below 150mg % (8.3 mM) after eating because of hypoglycaemic episodes. Hypoglycaemic coma occurs regularly in normal subjects whose blood sugar drops to 2 mM. As discussed earlier, (62, 63) in the presence of 5mM blood ketones there are no neurological symptoms when blood sugars fall to below 1 mM.

The present inventor has determined that supplementing type II diabetics with  
20 ketone bodies would allow better control of blood sugar, thus preventing the vascular changes in eye and kidney which occur now after 20 years of diabetes and which are the major cause of morbidity and mortality in diabetics.

Where the therapy is aimed at seizure related disorders, such as refractory epilepsy as is treated by the ketogenic diet, therapy is improved by use of ketone bodies, their  
25 polymers or esters or precursors such as butanediol compounds, due to the reduction or elimination of high lipid and carbohydrate content. Such patients include those with genetic defects in the brain glucose transporter system, in glycolysis or in PDH itself such as in Leigh's syndrome.

Particular disorders treatable with these medicaments are applicable to all  
30 conditions involving PDH blockage, including those conditions occurring after head trauma, or involving reduction or elimination of acetyl CoA supply to the mitochondrion such as

insulin coma and hypoglycaemia, defects in the glucose transporter in the brain or in glycolytic enzyme steps or in pyruvate transport.

Preferably the hydroxybutyrate is in the form of non-racemic D- $\beta$ -hydroxybutyrate and more preferably it is administered in a form which also supplies acetoacetate.

5 Preferably the metabolic precursor is one which when administered to a human or animal body is metabolised, eg. by liver, to produce one or both of D- $\beta$ -hydroxybutyrate and acetoacetate, more preferably in a physiological ratio. Particularly preferred are poly-D- $\beta$ -hydroxybutyric acid or acetoacetyl- $\beta$ -hydroxybutyrate oligomers or an ester of one or both of these. Lower alkyl esters such as C<sub>1-4</sub> alkyl esters may be employed but more preferably  
10 are more physiologically acceptable esters such as the respective 1,3-butandiol esters, particularly employing (R)-1,3-butandiol. Most preferred are the acetoacetyl- tri-, tetra- and penta- D- $\beta$ -hydroxybutyrate esters. Ester precursors will include esters of 1,3-butandiol, preferably (R) form and particularly acetoacetate esters such as acetoacetyl glycerol.

Preferred poly D- $\beta$ -hydroxybutyrate esters are those which are esters of the  
15 preferred oligomers of 2-100 repeats, eg. 2-20 repeats most preferably 2-10 repeats.

Where the medicament or nutritional product of the invention is for use without prolonged storage it is convenient to use it in the form of a liquid or solid composition comprising the hydroxy substituted carboxylic acid and/or the ketone, preferably comprising both and where these are the D- $\beta$ -hydroxybutyrate acids and acetoacetate  
20 together preferably in the ratio of about 3:1 to 5:1, more preferably about 4:1.

Where the medicament or aid comprises acetoacetate it is preferably not stored for a prolonged period or exposed to temperatures in excess of 40°C. Acetoacetate is unstable on heating and decomposes violently at 100°C into acetone and CO<sub>2</sub>. In such circumstances it is preferred that acetoacetate is generated by the composition on contact  
25 with the bodies metabolic processes. Preferably the composition comprises an ester precursor of actetoacetate. For example, the ethyl ester of acetoacetate is relatively stable with a boiling point of 180.8°C.

Still more preferably, the medicament or aid comprises an acetoacetyl ester of D- $\beta$ -hydroxybutyrate or such an ester of an oligomer of D- $\beta$ -hydroxybutyrate as described. This  
30 may be supplemented with D- $\beta$ -hydroxybutyrate or one of the polymers of that, e.g. oligo-D- $\beta$ -hydroxybutyrate, in order to bring about the preferred ratio of the two components.

Such a composition will provide the two preferred components when the ester and polymer are metabolised in the stomach or in the plasma of the human or animal which has consumed them. Again an (R) 1,3-butandiol ester of the acetoacetyl-D- $\beta$ -hydroxybutyrate may be most preferred as it will be more lipophilic until metabolised or otherwise  
5 deesterified and all its components are converted to the desired ketone bodies.

A second aspect of the invention provides novel esters of acetoacetate for use in therapy or as a nutritional aid. Such esters may include C<sub>1-4</sub> alkyl esters but most preferred are the D- $\beta$ -hydroxybutyryl-acetoacetate esters referred to above.

A third aspect of the present invention provides a poly-D- $\beta$ -hydroxybutyrate for use  
10 in therapy, particularly where this is in a form selected for its ability to be degraded in acid conditions of the stomach or by esterases in vivo.

A fourth aspect of the invention provides a method for the synthesis of D- $\beta$ -hydroxybutyryl-acetoacetate esters comprising the reaction of acetoacetic acid halide, e.g. acetoacetyl chloride, with D- $\beta$ -hydroxybutyrate. Preferably this is achieved by reacting  
15 acetoacetic acid with an activating agent, such as thionyl chloride, to produce the acid chloride.

A fifth aspect of the present invention provides a method for the synthesis of D- $\beta$ -hydroxybutyryl-acetoacetate esters comprising the reaction of D- $\beta$ -halobutyrate or its oligomers with acetoacetic acid, activated forms thereof or diketene .

20 A sixth aspect of the present invention provides a D- $\beta$ -hydroxybutyryl-acetoacetate ester per se, a physiologically acceptable salt or short or medium chain mono, di or trihydric alcohol or 1,3-butandiol ester thereof.

A seventh aspect of the present invention provides poly-D- $\beta$ -hydroxybutyrate together with a pharmaceutically or physiologically acceptable carrier.

25 An eighth aspect of the present invention provides a composition comprising D- $\beta$ -hydroxybutyrate and acetoacetic acid in a ratio of from 1:1 to 20:1, more preferably 2:1 to 10:1 and most preferably from 3:1 to 5:1 together with a pharmaceutically or physiologically acceptable carrier. Preferably the ratio of these components is about 4:1. Such a composition does not consist of plasma, serum or animal or plant tissue already used  
30 as a medicament or foodstuff, thus it is that which is preferably sterile and pyrogen free. Particularly the ketone bodies comprise at least 5% of the composition by weight, more

preferably 20% or more and most preferably 50% to 100%. The composition may be adapted for oral, parenteral or any other conventional form of administration.

A ninth aspect of the present invention comprises a method of treating a human or animal in order to increase their cardiac efficiency comprising administering to that person  
5 at least one of a materials for use in the first to eight aspects of the invention.

A tenth aspect of the present invention comprises a method of treating a human or animal in order to increase their the response to insulin comprising administering to that person at least one of a materials for use in the first to eight aspects of the invention.

An eleventh aspect of the present invention comprises a method of treating a human  
10 or animal in order to treat an insulin resistant state comprising administering to that person least one at least one of a materials for use in the first to eight aspects of the invention.

By insulin resistant state herein is included forms of diabetes, particularly those that do not respond fully to insulin.

A twelvth aspect of the invention provides a method of treating a human or animal  
15 in order to treat a nerve cell, eg. brain cell, death or damage related disorder as referred to for the first aspect, particularly a neurodegenerative disorder eg. such as those related to neurotoxic conditions such as presence of amyloid protein, eg. a memory associated disorder such as Alzheimer's disease, or epileptic seizures, comprising administering to that  
20 person least one at least one of a materials for use in the first to eight aspects of the invention.

Preferred methods of the ninth to twelvth aspects of the invention use the preferred ketones and polyacids and acid esters of the invention.

Methods of preparing poly D- $\beta$ -hydroxybutyrate are not specifically claimed as  
25 these are known in the art. For example Shang et al, (1994) Appli. Environ. Microbiol. 60: 1198-1205. This polymer is available commercially from Fluka Chemical Co. P1082, cat#81329, 1993-94, 980. Second St. Ronkonkoma NY 11779-7238, 800 358 5287.

Particular advantages of use of the biologically available polymers of the invention include the reduction in the amount of counter ions such as sodium that have to be  
30 coadministered with them. This reduction in sodium load is advantageous particularly in ill health. By biologically available is meant those materials which can be used by the

body to produce the least one of a D- $\beta$ -hydroxybutyrate, acetoacetate and a mixture of these in physiological ratio as described above

The amount of ketone bodies used in treatment of neurodegeneration such as Alzheimer's and Parkinsonism will preferably elevate blood levels to 0.5mM to 20mM, eg  
5 2mM to 7.5mM as described above. The present inventor estimates that 200 to 300g (0.5 pounds) of ketone bodies per patient per day might be required to achieve this. Where the treatment is through maintenance of cells against the effects of neurotox in this may be at a higher level, eg. 2 to 7.5mM in blood. Where it relies on the nerve stimulatory factor effect of the D- $\beta$ -hydroxybutyrate so produced the amount administered may be lower, eg.  
10 to provide 0.2 to 4 mM, but can of course be more for this or other disease.

It will be realised that treatment for neurodegenerative diseases such as Alzheimer's will most effectively be given soon after identifying patient's with a predisposition to develop the disease. Thus treatment for Alzheimers' most effectively follows a positive test result for one or more conditions selected from the group (i) mutations in the amyloid  
15 precursor protein gene on chromosome 21, (ii) mutations in the presenilin gene on chromosome 14, (iii) presence of isoforms of apolipoprotein E. Other tests shown to be indicative of Alzheimer's will of course be applicable.

Following such a positive test result it will be appropriate to prevent the development of memory loss and/or other neurological dysfunction by elevation of the total  
20 sum of the concentrations of the ketone bodies D- $\beta$ -hydroxybutyrate and acetoacetate in the patient's blood or plasma to say between 1.5 and 10 mM, more preferably 2 to 8mM, by one of several means. Preferably the patient is fed a diet of sufficient quantities of D- $\beta$ -hydroxybutyrate, its metabolisable polymers, its acetoacetate esters or their precursors (R)-1,3-butandiol and its acetoacetate esters, eg. acetoacetyl glycerol, or its administered  
25 intravenously or intrarterially the ketone bodies D- $\beta$ -hydroxybutyrate and acetoacetic acid. All of the organic materials referred to above are optionally in salt or ester form. Examples of typical physiologically acceptable salts will be selected from sodium, potassium, magnesium, L-Lysine and L-arginine or eg. more complex salts such as those of methyl glucamine salts.. Esters will be those as described previously for other aspects of the  
30 invention.

A still further aspect of the invention provides the ketone bodies of the invention

by suitable control of diet. Thus this aspect provides a method of treatment of a human or animal for a disorder of one or more of the ninth to the twelfth aspects of the invention comprising one of (i) total fasting of the individual and (ii) feeding the individual a ketogenic diet eg. of 60-80% lipid with carbohydrate content 20% or less by weight.

5 For the purpose of treating seizures, eg. in epilepsy, a diet may involve ad lib ingestion of carbohydrate by oral or enteral route or of the compounds specified above.

In all these treatments other than the ketogenic diet there is the improvement that a method of avoiding drop in blood ketones which accompanies the ingestion of excess carbohydrate and a method which avoids feeding of excess lipid which accelerates the  
10 synthesis by liver of fatty acids and cholesterol which would otherwise contribute to vascular disease.

It will be realised that hypoglycemic brain dysfunction will also be treatable using the treatments and compositions and compounds of the present invention. A further property associated with the present treatment will be general improvement in muscle  
15 performance.

The provision of ketone body based foodstuffs and medicaments of the invention is facilitated by the ready availability of a number of relatively cheap, or potentially cheap, starting materials from which (R)-3-hydroxybutyric acid may be derived (see Microbial Polyesters Yoshiharu Doi. ISBN 0-89573-746-9 Chapters 1.1, 3.2 and 8). The availability  
20 of genes capable of insertion into foodstuff generating organisms provides a means for generating products such as yoghurts and cheese that are enriched in either poly-(R)-3-hydroxybutyric acid or, after breakdown with enzymes capable of cleaving such polymers, with the monomeric substance itself (see Doi. Chapter 8).

The present invention will now be described further by way of illustration only by  
25 reference to the following Figures and experimental examples. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

## FIGURES

Figure 1 is a graph showing blood (R)-3-hydroxybutyrate level produced after time after gavage of (R)-3-hydroxybutyrate, an oligomer of this as produced in Example 1 and an acetoacetyl monomer thereof as produced in Example 2.

EXAMPLESEXAMPLE 1.10 Preparation of oligomers of (R)-3-hydroxybutyric acid (D-β-hydroxybutyrate).

(R)-3-hydroxybutyric acid (Fluka-5.0g: 0.048mole), p-toluene sulphonic acid (0.025g) and benzene (100ml) were stirred under reflux with a Dean-Stark trap arrangement for 24 hours. The reaction mixture was cooled and the benzene evaporated in vacuo (0.5mm Hg). 4.4g of colourless oil was obtained of which a 20mg sample was converted to the methyl ester for analysis of number of monomer repeats using NMR. These studies show that the product is a mixture of oligomers of D-β-hydroxybutyrate of average number of repeats 3.75, being mainly a mixture of trimers, tetramers and pentamers with the single most abundant material being the tetramer. The product mixture was soluble in 1 equivalent of sodium hydroxide.

20 EXAMPLE 2.Preparation of acetoacetyl ester of oligomeric (R)-3-hydroxybutyric acid.

A further batch of the colourless oil product from Example 1 (4.5g) was heated for 1 hour at 60°C with diketene (3.8g) and sodium acetate (0.045g) under nitrogen. Further diketene (3.8g) was added and the reaction heated for a further hour, cooled and diluted with ether, washed with water and then extracted with saturated sodium bicarbonate (5 x 100ml). Combined extract was washed with ether then acidified with concentrated HCl (added dropwise). Ethyl acetate extraction (3 x 50ml) was followed by drying over magnesium sulphate and evaporation in vacuo. A yellow solid/oil mixture was obtained (7.6g) which was chromatographed on a silica column using dichloromethane/methanol (98:2) to give a light amber oil product. Faster moving impurities were isolated (1.6g) and

after recolumning carbontetrachloride/methanol (99:1) 0.8g of oil was recovered which was shown by NMR and Mass spectrometry to be the desired mixture of acetoacetylated oligomers of D- $\beta$ -hydroxybutyrate. The product mixture had an Rf of 0.44 in dichloromethane/methanol (90:1) and was soluble in 1 equivalent of sodium hydroxide.

- 5 Both products of Example 1 and Example 2 are susceptible to separation of individual components by preparative HPLC.

### EXAMPLE 3.

#### Oral administration of D- $\beta$ -hydroxybutyrate, oligomers and acetoacetyl D- $\beta$ -hydroxybutyrate oligomers to rats.

- 10 The ability of orally administered D- $\beta$ -hydroxybutyrate and the oligomers of Examples 1 and 2 to raise blood ketone body levels was investigated as follows. Rats were starved overnight and then gavaged with 100  $\mu$ l/100g bodyweight of 4M D- $\beta$ -hydroxybutyrate brought to pH 7.74 using methyl glucamine. Blood levels of D- $\beta$ -hydroxybutyrate measured using and  
15 NAD<sup>+</sup>/EDTA assay of Anal. Biochem. 131, p478-482 (1983). 1.0ml of a solution made up from 2-amino-2-methyl-1-propanol (100mM pH 9.9, 0.094g/10ml), NAD<sup>+</sup> (30mM, 0.199g/10ml) and EDTA (4mM, 0.015g/10ml) was added to each of a number of cuvettes and 4 $\mu$ l sample or D- $\beta$ -hydroxybutyrate control.

- 20 As the rats had been fasted the initial levels of D- $\beta$ -hydroxybutyrate were elevated from the 0.1mM fed state. However, consistent serum increases of D- $\beta$ -hydroxybutyrate, between 1 and 3.2mM increase in each case, were provided.

- This procedure was repeated with 2M solutions of the mixtures of D- $\beta$ -hydroxybutyrate oligomers and their acetoacetyl esters described in Examples 1 and 2. The D- $\beta$ -hydroxybutyrate oligomer (19/1 in Figure 1) and the acetoacetyl ester (20/4 in Figure 1) were  
25 both brought to pH 7.6 with methyl glucamine and the blood D- $\beta$ -hydroxybutyrate level monitored using the aforesaid assay procedure. Increases in serum D- $\beta$ -hydroxybutyrate were shown to be of 0.5 to 1.2mM at 60 and 120 minutes after gavaging. These results demonstrate the efficacy of orally administered D- $\beta$ -hydroxybutyrate and its metabolic precursors of the invention in raising blood levels significantly for a period of hours after intake.

- 30 It was noted that the oligomeric esters 19/1 and 20/4, while not elevating the blood ketone body level as high as the monomer itself, did result in elevation for a much longer period of time and thus are suited to administration less frequently than the monomer.

## EXAMPLE 4.

**Table 2.** Sample 1500 calorie ketogenic diet using ketone bodies, their esters or polymers.

The ketones were assumed to contain 6 kcal/g, fats 9 kcal/g, carbohydrate and protein 4 kcal/g. Ketones have been substituted to give equivalent calories.

5

	Amount (g)	Fat (g)	Protein (g)	CHO (g)	Ketones (g)
<b>Breakfast</b>					
egg	32	4	4		
apple juice	70			7	
ketones	66				66
skim milk	92	0	2	3	
Total Breakfast		4	6	10	66
<b>Lunch</b>					
lean beef	12	1.75	3.5		
cooked carrots	45		0.6	3	
canned pears	40			4	
ketones	69.75				69.75
skim milk	92		2	3	
Total Lunch		1.75	6.1	10	69.75
<b>Supper</b>					
frankfurter	22.5	6	3		
cooked broccoli	50		1	2	
watermelon	75			5	
ketones	62.25				62.25
skim milk	92		2	3	
Total Supper		6	6	10	62.25
<b>Daily Total</b>		<b>11.75</b>	<b>18.1</b>	<b>30</b>	<b>198</b>

**EXAMPLE 5.****Effect of increased blood D- $\beta$ -hydroxybutyrate levels on whole brain GABA levels.**

To assess the effect of D- $\beta$ -hydroxybutyrate on whole brain GABA levels, and thus provide an indication of antiepileptic effect of ketone body or precursor treatment aimed at increasing blood ketone body levels, whole rat brain was frozen at set times after administration of D- $\beta$ -hydroxybutyrate as described in Example 3. GABA was assayed using standard HPLC technique and related to protein content using standard protein assay. At t=0 GABA levels were 191 pmoles/ $\mu$ g protein while at 120 minutes this was elevated at 466 pmoles/ $\mu$ g protein, demonstrating antepileptic potential.

10

**EXAMPLE 6****Methods****Culture Medium and Chemicals**

The serum free medium used from 0 to day 4 contained Neurobasal medium with B27 supplement diluted 50 fold (Life Technology, Gaithersburg, MD) to which was added: 0.5 mM L-glutamine, 25  $\mu$ M Na L-glutamate, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. After day 4, DMEM/F12 medium containing 5  $\mu$ M insulin, 30 nM l-thyroxine, 20 nM progesterone, 30 nM Na selenite 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin were used.

**20 Hippocampal Microisland Cultures**

The primary hippocampal cultures were removed from Wistar embryos on day 18 and dispersed by gentle agitation in a pipette. The suspension was centrifuge at 1,500 x g for 10 min and the supernatant discarded. New media was make 0.4-0.5 x 10<sup>6</sup> cells/ml. Ten  $\mu$ l of this suspension was pipetted into the center of poly D-lysine coated culture wells and the plates incubated at 38°C for 4 hrs and then 400  $\mu$ l of fresh Neurobasal media was added. After 2 days of incubation, half of the media was exchanged for fresh media and the incubation continued for 2 more days. After day 4, the medium was changed with DMEM/F12 medium containing 5  $\mu$ M insulin, 30 nM l-thyroxine, 20 nM progesterone, 30 nM Na selenite 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The wells were divided into 4 groups: half the wells received Na D- $\beta$ -hydroxybutyrate to a final concentration of 8 mM while and half of the wells received 5

25  
30

nM amyloid  $\beta_{1-42}$  (Sigma). These media were exchanged 2 days later (day 8) and the cells were fixed on day 10 and stained with anti MAP2 (Boehringer Mannheim, Indianapolis IN) to visual neurons and vimentin and GFAP (Boehringer) to visualize glial cells.

## 5 Results

### Cell Counts

Addition of D- $\beta$ -hydroxybutyrate to the incubation resulted in an increase in the neuronal cell number per microisland from a mean of 30 to mean of 70 cells per microisland. Addition of 5 nM amyloid  $\beta_{1-42}$  to the cultures reduced the cell numbers  
10 from 70 to 30 cells per microisland, confirming the previous observations of Hoshi *et al*, that amyloid  $\beta_{1-42}$  is toxic to hippocampal neurons. Addition D- $\beta$ -hydroxybutyrate to cultures containing amyloid  $\beta_{1-42}$  increased the cell number from a mean of 30 to 70 cells per microisland. From these data we conclude that addition of substrate level quantities of D- $\beta$ -hydroxybutyrate, to media whose major nutrients are glucose, pyruvate and L-  
15 glutamine, slows the rate of cell death in culture. We further conclude that D- $\beta$ -hydroxybutyrate can decrease the increased rate of hippocampal cell death caused by the addition of amyloid  $\beta_{1-42}$  in culture.

The number of dendritic outgrowths and the length of axons were both observed to have increased with presence of D- $\beta$ -hydroxybutyrate, whether  $\beta_{1-42}$  was present or  
20 not. This is indicative of nerve growth factor like behaviour.

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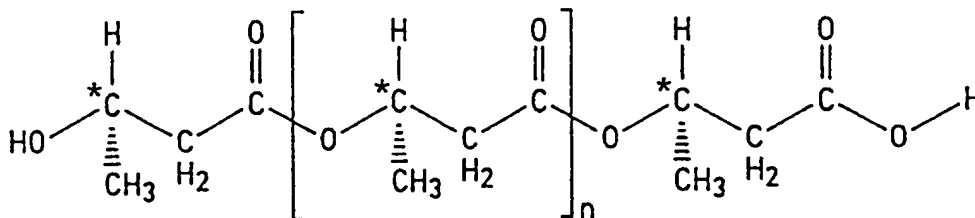
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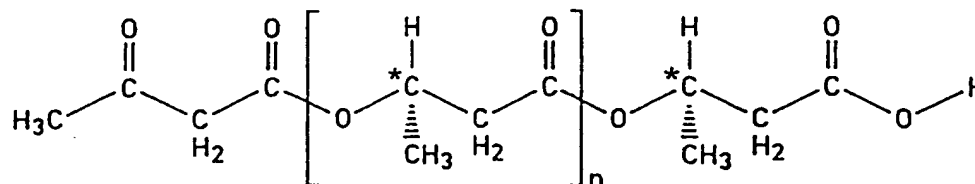
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CLAIMS.

1. The use of a compound selected from acetoacetate, D-β-hydroxybutyric acid and/or a metabolic precursor or physiological acceptable salt of either in the manufacture of a medicament or nutritional product
  - (i) for increasing cardiac efficiency
  - (ii) for treating diabetes and insulin resistant states and/or
  - (iii) for reversing, retarding or preventing the effects of neurodegenerative disorders and epilepsy.
2. Use as claimed in claim 1 wherein the neurodegenerative disorder is one associated with neurotoxic protein plaques.
3. Use as claimed in claim 1 wherein the metabolic precursor comprises (R)-1,3-butandiol, acetoacetyl or D-β-hydroxybutyryl moieties.
4. Use as claimed in claim 3 wherein the metabolic precursor is an ester.
5. Use as claimed in claim 4 wherein the esters are monohydric, dihydric or trihydric alcohol esters.
6. Use as claimed in claim 1 where the esters are acetoacetate esters.
7. Use as claimed in claim 1 where the esters are esters of D-β-hydroxybutyrate or esters of oligomers or polymers of D-β-hydroxybutyrate
8. Use as claimed in any one of the preceding claims wherein compounds are of formula



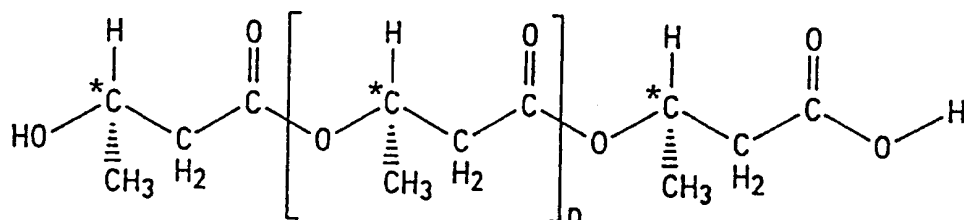
or



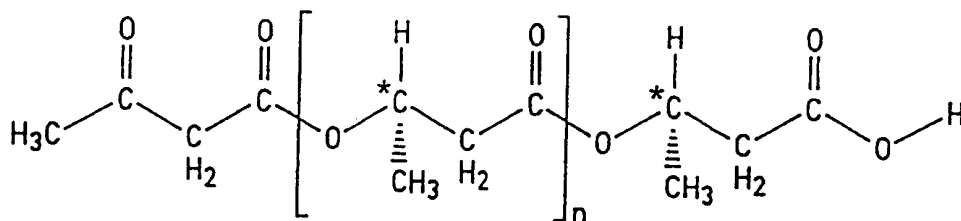
or physiological acceptable salts or esters thereof

wherein in each case n is selected such that the polymer or oligomer is readily metabolised on administration to a human or animal body to provide elevated ketone body levels in blood

- 5 9 Use as claimed in claim 8 wherein n is an integer of 0 to 1,000.
- 10 Use as claimed in claim 9 wherein n is an integer of 0 to 200.
- 11 Use as claimed in claim 10 wherein n is an integer of 3 to 5.
12. Use as claimed in claim 1 wherein the medicament provides D-β-hydroxybutyrate and acetoacetate in a ratio of 1:1 to 20:1.
- 10 13. A compound of formula



or



or physiological acceptable salts or esters thereof.

- 15 wherein in each case n is selected such that the polymer or oligomer is readily metabolised on administration to a human or animal body to provide elevated ketone body levels in blood.
14. A compound as defined in claim 13 wherein the esters are monohydric, dihydric or

## trihydric alcohol esters

15. A compound as claimed in claim 14 wherein the ester is of (R)-1,3-butandiol.
16. Poly-D- $\beta$ -hydroxybutyrate for use in therapy.
17. A method for the synthesis of D- $\beta$ -hydroxybutyryl-acetoacetate or poly or oligo-D- $\beta$ -  
5 hydroxybutyryl-acetoacetate esters comprising the reaction of acetoacetic acid halide  
with D- $\beta$ -hydroxybutyrate or poly- or oligo-D- $\beta$ -hydroxybutyrate.
18. A method as claimed in claim 17 comprising reacting acetoacetic acid with an activating  
agent to produce the acid halide.
19. A method for the synthesis of D- $\beta$ -hydroxybutyryl-acetoacetate esters comprising the  
10 reaction of D- $\beta$ -halobutyrate with acetoacetic acid.
20. A method for synthesis of D- $\beta$ -hydroxybutyryl-acetoacetate or oligo-  
D- $\beta$ -hydroxybutyryl-acetoacetate comprising reacting D- $\beta$ -hydroxybutyric acid with  
diketene.
21. A method as claimed in claim 20 wherein the reaction is carried out in the presence of  
15 sodium acetate.
22. A method as claimed in claim 21 wherein the reaction is carried out under nitrogen.
23. A composition comprising D- $\beta$ -hydroxybutyrate and acetoacetate in a ratio of from 1:1  
to 20:1, or respective metabolic precursors thereof providing such ratio on metabolism  
in vivo, optionally together with a pharmaceutically or physiologically acceptable  
20 carrier.
24. A composition as claimed in claim 23 wherein the ratio is from 3:1 to 5:1.
25. A method of treating a human or animal in order to increase their cardiac efficiency  
comprising administering to them D- $\beta$ -hydroxybutyric acid, optionally together with  
acetoacetate, or a metabolic precursor or physiological acceptable salt of either such as  
25 to elevate the blood level of said D- $\beta$ -hydroxybutyric acid.
26. A method of treating a human or animal in order to increase their the response to insulin  
comprising administering to them at least one of D- $\beta$ -hydroxybutyric acid, acetoacetate,  
or a metabolic precursor or physiological acceptable salt of either such as to elevate the  
blood level of D- $\beta$ -hydroxybutyric acid and/or acetoacetate.
- 30 27. A method of treating a human or animal in order to treat an insulin resistant state  
comprising administering to them at least one of D- $\beta$ -hydroxybutyric acid, acetoacetate,  
or a metabolic precursor or physiological acceptable salt of either such as to elevate the  
blood level of D- $\beta$ -hydroxybutyric acid and/or acetoacetate.

28. A method of treating a human or animal in order to treat a neurodegenerative disorder comprising administering to them at least one of D- $\beta$ -hydroxybutyric acid, acetoacetate, or a metabolic precursor or physiological acceptable salt of either such as to elevate the blood level of D- $\beta$ -hydroxybutyric acid and/or acetoacetate.
- 5 29. A method as claimed in any one of claims 19 to 21 comprising administering any one of the compounds described in claims 1 to 16.
30. A method as claimed in any one of claims 19 to 21 wherein the ketone bodies are provided by control of diet causing metabolic elevation of these compounds.
- 10 31. A method of synthesising an oligomer of D- $\beta$ -hydroxybutyric acid comprising heating a solution of D- $\beta$ -hydroxybutyric acid in a solvent until an oligomer of a desired number of repeats is produced.
32. A method as claimed in claim 31 wherein the desired number of repeats is from 2 to 10
33. A method as claimed in claim 32 wherein the solvent is aprotic.
- 15 34. A method as claimed in claim 33 wherein the solvent is aromatic.
35. A method as claimed in claim 34 wherein the reaction is carried out in the presence of an organic acid.
- 20 36. A method of treating a human or animal CNS cell, peripheral nerve cell, heart cell, or otherwise insulin insensitive cell in need of therapy for one or more of neurodegeneration, metabolic inefficiency or GABA preventable seizure, or inability to metabolise glucose, comprising administering to that cell at least one of D- $\beta$ -hydroxybutyric acid, acetoactate, or a metabolic precursor or physiological acceptable salt of either

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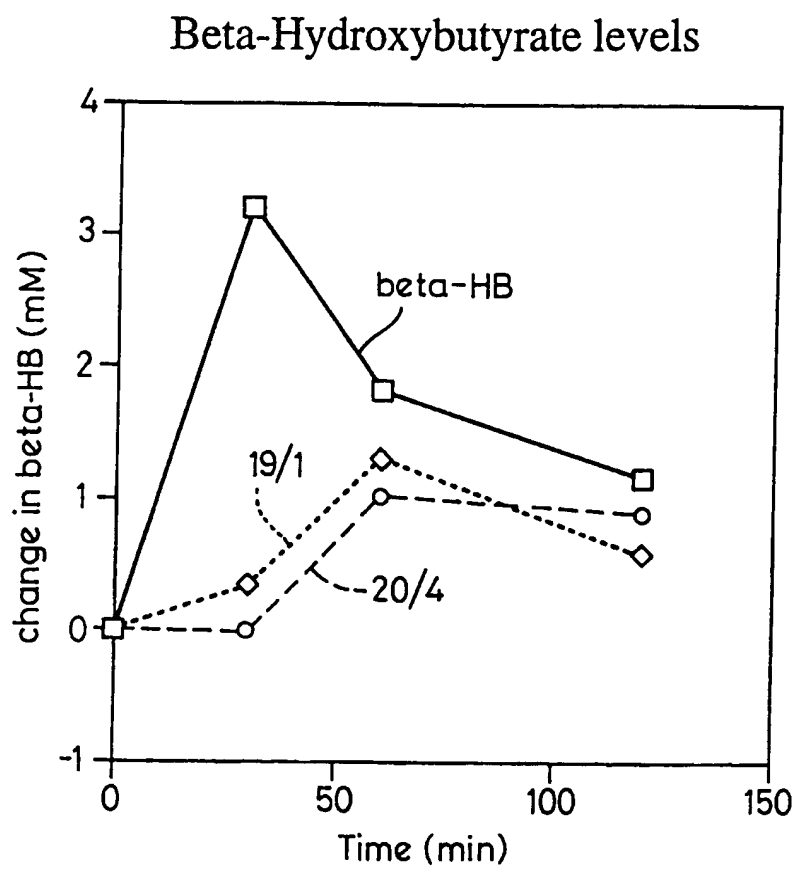


Fig.1

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/05072

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/19, 31/22, 31/225

US CL : 514/547, 557, 574

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/547, 557, 574

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE - structure search.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 5,719,119 A (VEECH) 17 February 1998, see abstract.	1-36
X	US 5,116,868 A (CHEN et al.) 26 May 1992, see abstract.	1-36
X	US 5,112,865 A (NICHELS et al.) 12 May 1992, see abstract.	1-36
X	US 4,346,107 A (CAVAZZA et al.) 24 August 1982, see abstract.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 MAY 1998

Date of mailing of the international search report

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